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PROTON-TRANSLOCATING CYTOCHROME *c* OXIDASE IN ARTIFICIAL PHOSPHOLIPID VESICLES

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Summary

The proton translocating properties of cytochrome *c* oxidase have been studied in artificial phospholipid vesicles into the membranes of which the isolated and purified enzyme was incorporated.

Initiation of oxidation of ferrocytochrome *c* by addition of the cytochrome, or by addition of oxygen to an anaerobic vesicle suspension, leads to ejection of H^+ from the vesicles provided that charge compensation is permitted by the presence of valinomycin and K^+. Proton ejection is not observed if the membranes have been specifically rendered permeable to protons.

The proton ejection is the result of true translocation of H^+ across the membrane as indicated by its dependence on the intravesicular buffering power relative to the number of particles (electrons and protons) transferred by the system, and since it can be shown not to be due to a net formation of acid in the system.

Comparison of the initial rates of proton ejection and oxidation of cytochrome *c* yields a H^+/e^- quotient close to 1.0 both in cytochrome *c* and oxygen pulse experiments. An approach towards the same stoichiometry is found by comparison of the extents of proton ejection and electron transfer under appropriate experimental conditions.

It is concluded that cytochrome *c* oxidase is a proton pump, which conserves redox energy by converting it into an electrochemical proton gradient through electrogenic translocation of H^+.

Introduction

Based on work in our laboratory, we have proposed that mitochondrial cytochrome *c* oxidase (ferrocytochrome *c* : oxygen oxidoreductase, EC

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MOPS, 4-(morpholino)-propanesulfonic acid; FCCP, *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone.

1.9.3.1.) functions as a redox-linked proton pump [1—4]. Thus the transfer of electrons from cytochrome *c* to oxygen was proposed to be obligatorily linked to vectorial translocation of H^+ out across the mitochondrial cristae membrane with conservation of the released redox energy as an electrochemical proton gradient. This is consistent with the most central idea of the chemiosmotic theory [5]. The stoichiometry of the transport process was found to be 1 H^+/e^- both in intact mitochondria and in sonicated submitochondrial particles (in the latter, the membrane is "inverted" and proton transport has a direction opposite to that in mitochondria). Due to the necessary protonation of reduced oxygen to form water, it was concluded that two electrical charges are separated across the membrane per transferred electron in the cytochrome oxidase reaction (see also Fig. 1B). This charge translocation stoichiometry is also supported by direct experiment [4].

These findings are incompatible with the model of cytochrome oxidase function as previously proposed by Mitchell [6,7] and supported by others [8—11] (see Fig. 1A). According to this model, cytochrome oxidase simply transfers electrons vectorially across the membrane with development of a membrane potential and a pH gradient, the latter due to consumption of the protons required in water formation from the matrix (M-) phase. No protons are ejected into the C-phase and the stoichiometry of charge translocation is one charge equivalent per electron (Fig. 1A).

In this work a study of the direction of electron transport with respect to the membrane was not specifically attempted (but see refs. 1—4). Instead, we have tried to distinguish between the two models by experiments specifically designed to test for the presence (Fig. 1B) or absence (Fig. 1A) of proton transport, i.e. their most fundamental difference. The models in Fig. 1 have therefore not been made to differ with respect to the direction of electron

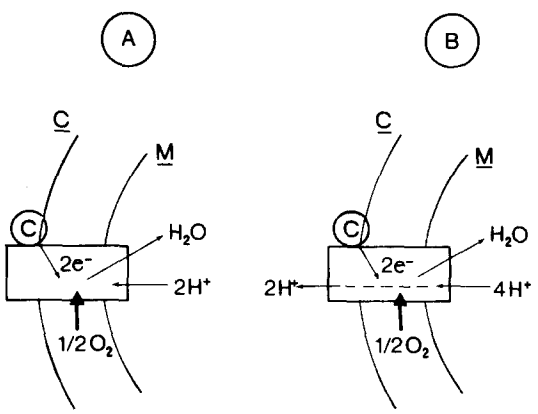

Fig. 1. Two models of the bioenergetic function of cytochrome *c* oxidase. The mitochondrial cristae membrane is depicted as a curved area surrounded by the cytoplasmic (C) space to the left (more correctly, the intermembrane region) and the inner mitochondrial matrix (M) space to the right. The rectangular structure depicts the membraneous cytochrome oxidase and the encircled C, cytochrome *c*. The overall redox reaction (Eqn. 1) is depicted by arrows leading to and from the "centre" of the rectangula. In A, the only particular aspect of this reaction is the uptake of H^+ required in water formation from the M-space. In B, the redox reaction is in addition linked to electrogenic transport of $2H^+$ per $2e^-$ from the M- to the C-space. For further details, see text.

transfer (but see refs. 1—4), but with respect to true proton transport only. To make this distinction we have studied the cytochrome oxidase reaction with the enzyme incorporated into artificial phospholipid vesicles using a quantitative approach. Previously reported qualitative data [3] with cytochrome oxidase vesicles favoured scheme B over scheme A in Fig. 1.

Materials and Methods

Cytochrome *c* oxidase was isolated and purified from beef heart mitochondria [12] essentially as described by Kuboyama et al. [13], but using cholate alone as detergent (see also ref. 14). The final haem/protein ratio of the preparation was 9—11 nmol/mg protein, using an extinction coefficient of 13.5 $mM^{-1} \cdot cm^{-1}$ for reduced minus oxidised haem at the wavelength couple 605 minus 630 nm. The preparations were pure as judged from polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, and comparable in this respect to preparations previously described (see e.g. ref. 15).

Cytochrome oxidase vesicles were prepared using the method described by Hinkle et al. [16] with some modifications. 100 mg of crude soybean phospholipids (Asolectin) were dispersed in 2.5 ml solution usually containing 40 mM KH_2PO_4, 40 mM Tricine (*N*-tris(hydroxymethyl)methylglycine), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonate) and 1—1.5% (w/v) potassium cholate, pH 7.5. The cholate was recrystallised before use (see below). The solution was subjected to sonic oscillations (Branson sonifier) until clarified. Then either 12 μl, but usually 120 μl of purified cytochrome *c* oxidase (30 mg protein/ml) was added, and the solution immediately dialysed for 4 h against 0.5—1.0 l of buffer with the same constituents as above, but without cholate. Dialysis was continued overnight against 1 l of 220 mM sucrose supplemented with 0.5 mM HEPES and Tricine respectively, pH 7.5. The dialysis was performed at 4° C.

In many experiments MOPS (4-(morpholinopropane)sulphonic acid) was substituted for HEPES, and either MOPS or HEPES substituted for KH_2PO_4, with little effect on the results. In some experiments the intravesicular buffering capacity was varied, in which case the appropriate conditions are given in the figure legend.

The cytochrome oxidase vesicles exhibited respiratory control as judged from stimulation of oxygen consumption (measured polarographically in the presence of 6.7 mM ascorbate and 75 μM cytochrome *c*) by addition of either 5 μM FCCP (*p*-fluoromethoxycarbonylcyanide phenylhydrazone) plus valinomycin (1 μg/ml), or valinomycin plus nigericin (0.1 μg/ml). Based on the respiratory control quotient, which was usually about 4—5 (see refs. 8, 16), the vesicles remained intact for at least one week when stored on an ice-water bath.

Reduced cytochrome *c* was prepared as described previously [3] and the exact concentration was determined by measuring oxidation of the cytochrome at 550 nm with an excess of ferricyanide, using an extinction coefficient (reduced minus oxidised) of 21 $mM^{-1} \cdot cm^{-1}$ [17]. The preparation contained less than 5% oxidised cytochrome as determined by reduction with dithionite.

pH changes associated with the function of the cytochrome oxidase vesicles were measured either electrometrically or spectrophotometrically. A Beckman or Ingold pH combination electrode was used in conjunction with a pH meter (Instrulab IM-555), connected to a Goerz Servogor RE 541 strip chart recorder. Open vessels were used with vigorous magnetic stirring either at room temperature (22° C) or thermostated at a temperature specified in the figure legend. pH changes were calibrated with standard HCl and oxalic acid solutions. The response time of the entire system was 1—2 s (50%) at room temperature. This was found to be sufficient for static but not for kinetic measurements of cytochrome oxidase vesicles (see Results).

Kinetic measurements of changes in pH and the redox state of cytochrome *c* were therefore done either with an Aminco DW2 spectrophotometer or a dual-wavelength spectrophotometer (model DBS-1) built and designed at the Johnson Research Foundation, Philadelphia. The latter instrument was used in conjunction with a Johnson Foundation rapid flow apparatus (model E, ref. 18). pH was measured either with phenol red or bromocresol purple as indicators [19] at wavelength couples carefully chosen not to show any significant absorption changes due to oxidoreduction of cytochrome *c* (see figure legends). Absorption changes due to redox transitions in cytochrome aa_3 were negligible due to the generally very low concentration of enzyme and to the choice of wavelengths.

The side-syringe of the flow apparatus (mixing ratio 1 : 80) contained unbuffered air-saturated salt solution, the pH of which was adjusted to be approximately the same as the contents of the main syringes. Reduction of cytochrome *c* (measured at 550—541 nm) indicated anaerobiosis of the cytochrome oxidase vesicle suspension. The time after mixing (time from mixing to observation) of air-saturated medium with the vesicle suspension was generally about 10 ms. The light-path of the flow apparatus is 0.6 cm, in all other spectrophotometric experiments it was 1 cm.

Protein was determined by the Folin procedure [20] with human serum albumin as standard. The cholate used in the isolation of cytochrome aa_3 and preparation of vesicles was recrystallised as follows. 25 g of cholic acid (Merck, Darmstadt, G.F.R.) were dissolved in 1 l ethanol. Approx. 1 g of activated carbon (Darco, Wilmington, Del.) was added and the solution was incubated for 1—2 h, filtrated until clarified, and concentrated by destillation to a volume of 0.3 l. Then water was added to a final volume of 1 l, and the cholate was crystallised overnight at 4° C, and finally washed with diethyl ether.

All reagents were of highest purity available commercially. FCCP was a gift from Dr. P.G. Heytler. Nigericin was obtained through the courtesy of Dr. Jorma Järvisalo.

Results

The addition of ferrocytochrome *c* to aerobic suspensions of cytochrome oxidase vesicles initiates respiration, and as shown in Fig. 2 (left), this is initially associated with a rapid acidification of the extravesicular medium (see also ref. 3). Following the acidification phase, steady proton consumption

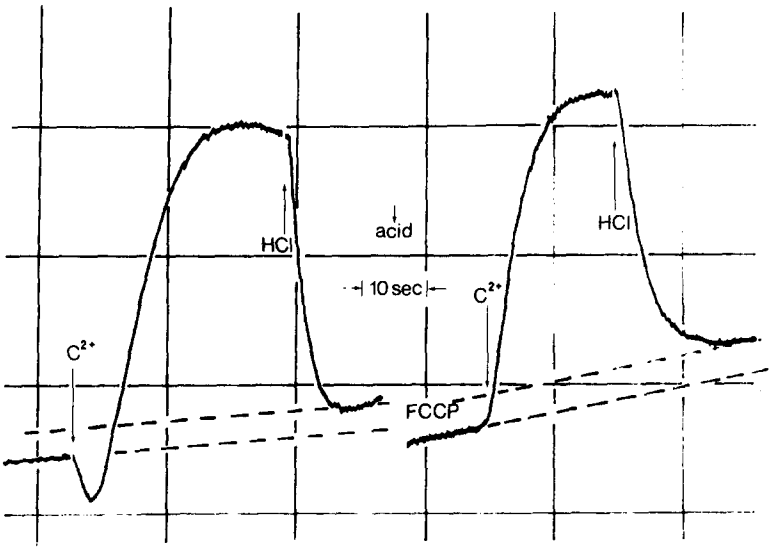

Fig. 2. Redox-linked proton ejection from cytochrome oxidase vesicles. The reaction mixture contained 50 mM K_2SO_4, 1 μg/ml valinomycin and 40 μl of cytochrome oxidase vesicles (standard preparation, see Materials and Methods) in a final volume of 2.0 ml. Temperature, 22°C. pH is recorded electrometrically (ordinate) with respect to time (left to right). At the arrow denoted C^{2+} 8.5 nequiv. ferrocytochrome *c* is added. Standard HCl is added to an amount of 7 nequiv. pH is then adjusted by an aliquot of KOH, FCCP (6 μM) is added, and the experiment is repeated. The change in pH on addition of the standard HCl corresponds to approx. 0.04 pH units.

ensues as expected from the overall reaction

$$2 \text{ cytochrome } c^{2+} + \tfrac{1}{2}O_2 + 2\ H^+ \rightarrow 2 \text{ cytochrome } c^{3+} + H_2O \qquad (1)$$

until all the added cytochrome *c* is oxidised. In the presence of a proton-conducting uncoupling agent, however (FCCP, Fig. 2, right trace), the cytochrome *c* addition is directly followed by proton consumption without the acidification phase. The latter was likewise abolished in the presence of nigericin [3], or in conditions where the potassium ionophore valinomycin was left out of the reaction mixture (see below, Fig. 4B).

The overall consumption of protons linked to oxidation of cytochrome *c* by oxygen is defined by Eqn. 1 as 1 H^+ consumed per electron transferred. With due account of the slight drifts in the pH traces (dashed lines, Fig. 2), the observed overall H^+/e^- quotients of proton consumption in the absence and presence of FCCP amount to 0.95 and 0.93, respectively, as measured using the HCl calibration and the number of added equivalents of ferrocytochrome *c*. It is hence apparent that the measured overall stoichiometry of H^+ consumption is unaffected by the uncoupling agent, and that it is the same whether the acidification phase occurs or not. The acidification phase, which in this experiment amounts to at least 1.2 equiv. H^+ (but see below for larger extents of H^+ ejection), is therefore clearly not due to net production of H^+ in the system that would persist after completion of the overall reaction (Eqn. 1). This property of the acidification effect (see also ref. 3), together with its sensitivity towards uncoupling agents and valinomycin, strongly suggests that it is the result of proton transport out across the vesicle membrane (see Discussion),

and is qualitatively in accordance with Fig. 1B.

Control experiments under conditions similar to those in Fig. 2 have indicated that the acidification phase is truly dependent upon cytochrome *c* oxidase activity. Thus it is blocked by cyanide, does not occur on pulsing with oxidised rather than with reduced cytochrome *c*, or on adding reduced cytochrome *c* to phospholipid vesicles without the inlayed enzyme (not shown). Moreover, an increase in the amount of cytochrome aa_3 at constant phospholipid concentration (the enzyme/phospholipid ratio has been changed 10-fold this way, see Materials and Methods), which leads to a larger population of enzymatically active vesicles as long as a critical enzyme/phospholipid ratio is not exceeded, was found to result in a profound increase in the extent of the acidification phase. Hence the acidification is certainly not due to a trivial ionic interaction between ferrocytochrome *c* and phospholipids.

Our pH electrode system does not respond fast enough to accurately measure the kinetics of the acidification phase, not even at temperatures close to 0°C. However, at such temperatures the turn-over of the enzyme was sufficiently slowed to permit kinetic studies with a pH indicator using conventional manual stirring. Fig. 3 shows an example of such an experiment. From comparison of the initial rates of proton appearance (lower trace) and oxidation of cytochrome *c* (uppper trace), H^+/e^- stoichiometries of proton ejection very close to 1.0 were obtained (see also below). This is the same stoichiometry

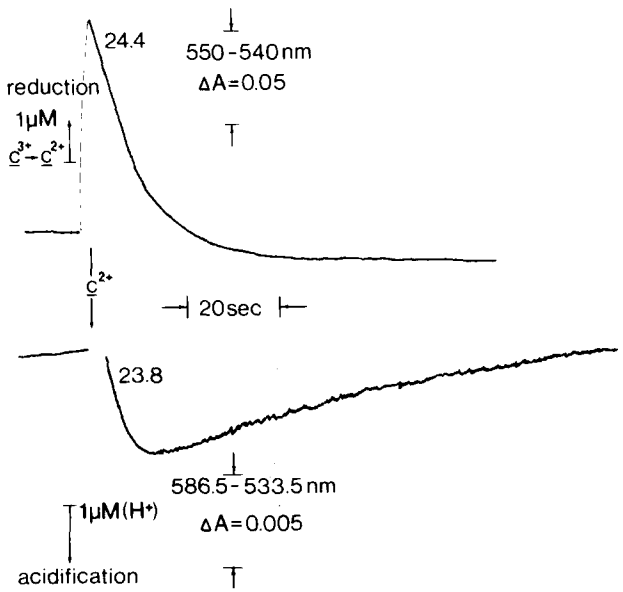

Fig. 3. Proton ejection and cytochrome *c* oxidation at low temperature. The reaction mixture contained 50 mM K_2SO_4, 0.8 μg/ml valinomycin, 16 μM bromocresol purple (lower trace only) and 0.2 ml cytochrome oxidase vesicles (P_i/MOPS/Tricine, see Materials and Methods) in a final volume of 3.2 ml, pH 6.6. Temperature, 3°C. At the arrow marked c^{2+} 5 μl of 1.54 mM ferrocytochrome *c* solution are stirred into the cuvette, and the redox state of the cytochrome (upper trace) and ionisation state of the pH indicator (lower trace) are measured respectively as functions of time. The numbers adjacent to the traces represent the initial rates of oxidation and acidification respectively, expressed as μM/min of cytochrome *c* oxidised and H^+ ions released.

as previously found in intact mitochondria and in sonicated submitochondrial particles [1—4], and agrees quantitatively with Fig. 1B. The pH trace of Fig. 3 also shows an interesting phenomenon, to be described in detail below, viz. that the alkalinisation phase following the initial acidification is very far from completion at the point where cytochrome *c* is already fully oxidised.

As shown in the experiment of Fig. 4A, which was performed at 26°C, the addition of ferrocytochrome *c* to the cytochrome oxidase vesicle suspension results in a large (11 nequiv. H^+) acidification phase, which is followed only by very slow drift towards alkalinisation (contrast Fig. 2), although the redox reaction was completed within 20 s (not shown, see Fig. 3). However, when nigericin (Fig. 4A) or an uncoupling agent (not shown) is added, the alkalinisation proceeds quickly to the equilibrium position defined for the overall process by Eqn. 1. Thus the H^+/e^- quotient of overall proton consumption from the point prior to addition of cytochrome *c* to the final state equilibrated with nigericin, is 1.00 in Fig. 4A allowing for the slight drift in the pH trace (dashed line). Very similar findings were made in experiments where nigericin was added prior to cytochtome *c*. As already pointed out above

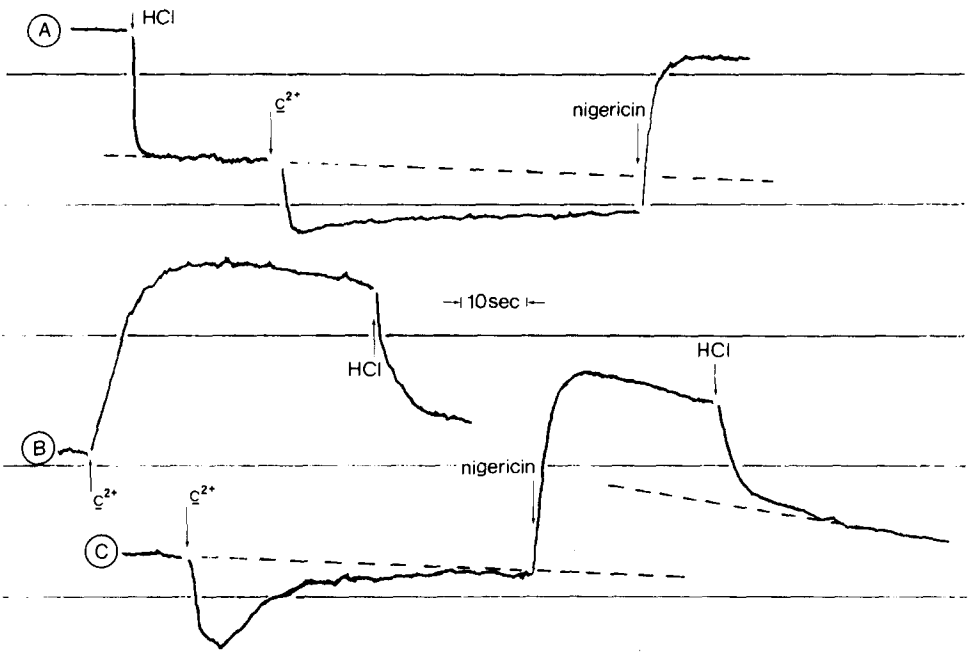

Fig. 4. Proton ejection from cytochrome oxidase vesicles. Effect of valinomycin, nigericin and vesicle concentration. Reaction mixture: 50 mM K_2SO_4, 0.8 μg/ml valinomycin (except in B) and either 0.2 ml of cytochrome oxidase vesicles in a final volume of 3.3 ml (B and C), or 0.5 ml of vesicles in a final volume of 3.0 ml (A). The vesicles were prepared with an inside milieu of P_i/MOPS/Tricine (see Materials and Methods). In A, the following additions were made as indicated: 20 nequiv. HCl, 19.2 nequiv. ferrocytochrome *c* and 0.5 μg nigericin. In B, valinomycin was absent, and 19.2 nequiv. ferrocytochrome *c* and 10 nequiv. HCl were added as indicated. Then 2.5 μg valinomycin were added and the pH was readjusted (not shown). C shows the continuation of this experiment with additions of 19.2 nequiv. ferrocytochrome *c*, 0.5 μg nigericin and 10 nequiv. HCl as indicated. pH is recorded electrometrically as a function of time (from left to right). A downward deflection denotes acidification, the extent of which is defined by the standard HCl additions. These resulted in a pH shift of approx. 0.02 units. Temperature, 26°C. pH 7.15—7.20.

(cf. description of Fig. 2), it is clear that the acidification phase is not due to net production of acid in the system which would persist in the final equilibrium state. Hence Fig. 4A suggests that the cytochrome *c* oxidase reaction is linked to an acidification of the extravesicular space, which in this particular experiment amounts to a H^+/cytochrome aa_3 ratio larger than 10, that the redox reaction may proceed to completion under conditions where little H^+ consumption, as demanded by the overall process (Eqn. 1), is observed extravesicularly, and that the proton-linked component of the overall process can be observed quantitatively in the extravesicular space only when the vesicle membrane has been rendered permeable to H^+ ions. These findings strongly suggest that the acidification phase is a consequence of true proton transport across the vesicle membrane, and that the protons required for formation of water (Eqn. 1) are also taken from the intravesicular space, in accordance with Fig. 1B.

Fig. 4B shows that the acidification phase is not observed if valinomycin is left out of the reaction mixture, the pH response on addition of ferrocytochrome *c* being very similar to the responses in the presence of an uncoupler. This important finding suggests that the proton ejection phase is dependent upon discharge of the electrical membrane potential by valinomycin plus K^+ (see Discussion).

The experiment of Fig. 4B is continued in Fig. 4C after addition of valinomycin and readjustment of pH (not shown). This experiment (Fig. 4B, C) is performed with the same preparation of vesicles as the experiment of Fig. 4A, but at a nearly three times lower concentration. In Fig. 4C it may be seen that the acidification phase following addition of cytochrome *c* is now more "unstable" with a much faster partial relaxation than was the case in Fig. 4A. In all other respects this experiment appears very similar to that in Fig. 4A. Thus the "stability" of the acidification phase may be enhanced by increasing the concentration of cytochrome oxidase vesicles keeping all other parameters constant.

The more systematical series of experiments shown in Fig. 5 demonstrates that the "stability" of the acidification phase is, in fact, dependent upon two major conditions, (i) the intravesicular buffering capacity, and (ii) the concentration of added cytochrome *c*. As shown in Fig. 5, this dependence is such that the acidification phase becomes more stable the higher the intravesicular buffering power, and the lower the concentration of added cytochrome *c*. This finding is equivalent to the one made by comparing Fig. 4A and C above, since as the vesicle concentration increases (at constant concentration of added cytochrome *c*), the number of turn-overs per vesicle decreases. This is effectively the same as a decrease in the number of consumed reducing equivalents per vesicle (at constant intravesicular buffer capacity per vesicle), leading to a more stable acidification phase (cf. Fig. 5). It is concluded that these properties of the acidification phase are exactly those expected from a redox-linked proton transport process (see Discussion).

Table I summarises calculated apparent H^+/e^- quotients for the proton ejection observed under the different conditions in Fig. 5. It should be noted that this apparent H^+/e^- quotient is higher the more "stable" the acidification phase, and yields a maximum value of 0.71 under the most favourable con-

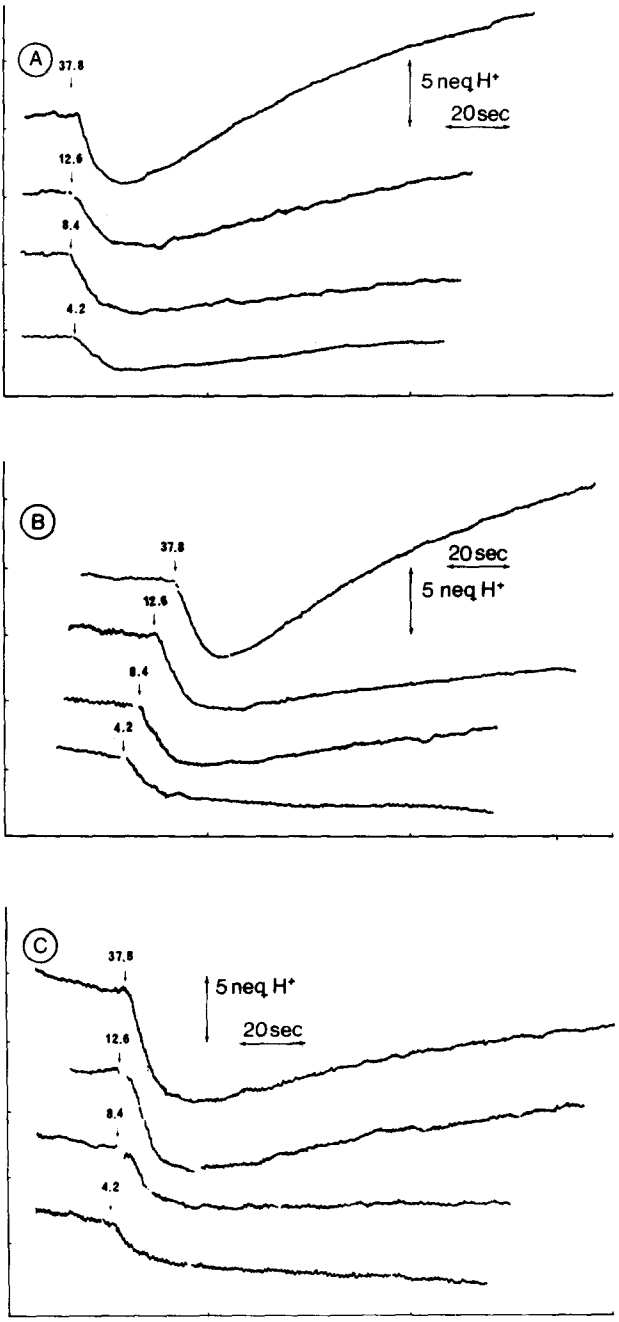

Fig. 5. The effect of inside buffer capacity and extent of redox reaction on proton ejection by cytochrome oxidase vesicles. The reaction mixture contained 50 mM K_2SO_4, 0.8 μg/ml valinomycin and 0.2 ml of cytochrome oxidase vesicles in a final volume of 3.2 ml. Temperature, 2°C, pH 6.7. The vesicles used in A, B and C differed only with respect to the composition of the intravesicular space. In A this consisted of 10 mM KH_2PO_4, 10 mM MOPS, 2.5 mM Tricine and 67.5 mM KCl. In B these constituents were added at concentrations of 20, 20, 5 and 45 mM, respectively, and in C 40, 40 and 10 mM without added KCl. In all other respects the preparations were made as described in Materials and Methods. They also had similar respiratory rates and respiratory control quotients. The arrows show the point of addition of ferrocytochrome *c* (nequiv.). pH is measured electrometrically as in Figs. 2 and 4.

TABLE I

H^+/e^- QUOTIENTS OF PROTON TRANSPORT AS CALCULATED FROM REACTION EXTENTS

The data from Fig. 5 were treated as follows: The intercept of the straight lines extrapolated from the maximal rates of H^+ consumption and production was taken as the point of maximum H^+ production. The distance from this point to the extrapolated pH baseline (before cytochrome *c* addition) was taken as ΔH^+. ΔH^+ was divided by the amount of added cytochrome *c* to yield the calculated apparent H^+/e^- quotients listed below.

Intravesicular buffer strength	Added cytochrome c (nequiv.)			
	37.8	12.6	8.4	4.2
Low (Fig. 5A)	0.19	0.39	0.58	0.65
Medium (Fig. 5B)	0.19	0.45	0.58	0.68
High (Fig. 5C)	0.24	0.68	0.52	0.71

ditions. As discussed below, these conditions come close to a situation where an estimation of the true H^+/e^- quotient of redox-linked proton transport from the extents of H^+ ejection and cytochrome *c* oxidation may become permissible.

The coupling of proton ejection (transport) to electron transfer may also be studied by initiating the reaction with oxygen under initially anaerobic conditions. In these experiments, we employed the pH indicators phenol red and bromocresol purple, the different p*K* values of which [19] permitted studies both above and below pH 7, respectively. A rapid flow apparatus was used to study the initial kinetics of both proton appearance and consumption, and also of oxidation of cytochrome *c*. This method has the advantage of kinetic compatibility and of removing possible artifacts that might arise from the addition of cytochrome *c*. In the presence of cytochrome oxidase vesicles, cytochrome *c* and ascorbate, the anaerobic state was generated subsequent to each pulse of oxygen so that several consecutive O_2 pulses could be given to the same vesicle suspension allowing a thorough analysis. Controls were always included in which a high concentration of buffer was added to the system to test for the extent by which the indicators responded to changes other than H^+ concentration. In most cases these controls indicated no or very small absorbance changes, mainly due to very careful choice of wavelength couples in pilot experiments.

The oxidation of ascorbate by ferricytochrome *c* leads to release of protons. However, during the initial stages of O_2-initiated reaction with reduced cytochrome oxidase vesicles (0.01—3 s), there was little if any, oxidation of ascorbate. Separate experiments performed under identical conditions, but measuring the rate of reduction of cytochrome *c* by ascorbate (not shown), then proved that the second order rate constant for reduction of cytochrome *c* by ascorbate is about 5 $M^{-1} \cdot s^{-1}$ at 22°C, probably due to the high ionic strength of our experimental media (cf. ref. 21). Consequently, this reaction is extremely slow in comparison with the rate of oxidation of cytochrome *c* following the pulse of oxygen. In fact, the $t_{1/2}$ for the former reaction is longer than 25 s under our experimental conditions. Therefore, there is insignificant oxidation of ascorbate in the measured initial stages of the reaction of O_2 with

cytochrome oxidase vesicles, and hence, any observed production of H^+ can not arise from that particular reaction.

Figs. 6 and 7 show the changes in $[H^+]$ and oxidation of cytochrome *c*, respectively, on initiation of respiration of cytochrome oxidase vesicles by oxygen. In Fig. 6, ejection of protons is seen (measured here with bromocresol purple) following a mixing artifact that occurs during the flow, not being linked to any significant oxidation of cytochrome *c* (cf. Fig. 7). H^+ ejection (Fig. 6A) is followed by consumption, whereas H^+ consumption starts immediately in the presence of nigericin (Fig. 6B), or an uncoupling agent (not shown), confirming the data obtained with ferrocytochrome *c* pulses (Figs. 2—5). The control shown in Fig. 6C, in the presence of a high concentration of buffer, proves that the pH indicator is largely independent of changes other than in H^+ concentration under the experimental conditions. Comparison of the initial rate of proton ejection in Fig. 6A with the initial rate of oxidation of cytochrome *c* (Fig. 7) yields a H^+/e^- quotient of proton ejection very close to 1.0 (see figure legend), in excellent agreement with the cytochrome *c* pulse experiments (Fig. 3). The H^+/e^- stoichiometry of proton consumption under uncoupled conditions is also very close to the expected value of 1.0 according to Eqn. 1 (cf. Figs. 6B and 7, see legends). The results obtained with the pH indicator technique were not appreciably different at pH 6.5 (Figs. 6 and 7) and pH 7.5 (not shown) using bromocresol purple and phenol red, respectively.

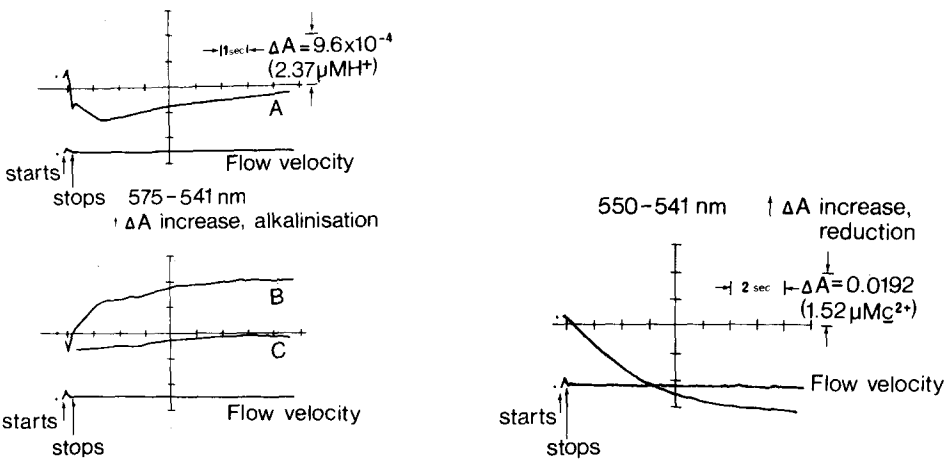

Fig. 6. Proton ejection and consumption by cytochrome oxidase vesicles initiated by oxygen. The reaction mixture contained 50 mM K_2SO_4, 0.05 mM MOPS, 3.3 mM potassium ascorbate, 6 μM cytochrome *c*, 0.33 μg/ml valinomycin, 20 μM bromocresol purple and 1.75 ml cytochrome oxidase vesicles (standard preparation, see Materials and Methods) in a final volume of 15 ml, pH 6.6. Temperature, 22°C. In B a further addition of 1 μg/ml of nigericin was made, and C was performed in the presence of nigericin plus 15 mM HEPES, pH 7.0. Oxygen is added from the side-syringe of the rapid flow apparatus (see Materials and Methods) in the form of air-saturated 50 mM K_2SO_4 to a final concentration of approx. 4 μM O_2. The reaction is followed after the flow stops (approx. 10 ms from mixing) indicated by the flow velocity traces. In A the initial rate of H^+ ejection after the flow stops if 0.87 μM/s. In B the rate of H^+ consumption is 0.85 μM/s. For comparison of A with the rate of electron transfer, see Fig. 7.

Fig. 7. Oxidation of cytochrome *c* initiated by oxygen in cytochrome oxidase vesicles. Experimental conditions exactly as described for Fig. 6. The initial rate of cytochrome *c* oxidation after the flow stops is 0.82 μM/s which may be compared with the rates of proton ejection and consumption in Fig. 6A.

Discussion

The oxidation of added ferrocytochrome *c* by cytochrome oxidase vesicles is associated with an initial phase of H^+ release into the aqueous medium (ref. 3, Figs. 2—6), although the overall process of oxidation of cytochrome *c* by oxygen demands consumption of 1 H^+ ion per transferred electron (see Eqn. 1). One of the most striking properties of the acidification phase is its sensitivity to uncoupling agents or nigericin (in the presence of valinomycin), which effectively render the vesicle membranes permeable to H^+ ions. This property strongly suggests that the H^+ ejection phase is a consequence of proton transport across the membrane.

If the acidification phase were the result of net acid production in the system (medium plus vesicles), it should not "vanish" under conditions which permit equilibration of H^+ ions across the membrane, but should show up as a deviation in the overall H^+/e^- quotient of proton consumption (Eqn. 1) to lower values. As shown in Figs. 2 and 4, the overall stoichiometry of proton comsumption is completely independent of whether the H^+ ejection phase has occurred (no uncoupling agent) or not (plus uncoupler). This provides strong evidence that the H^+ ejection is indeed the result of a shift (or transport) of H^+ ions from one phase (the aqueous intravesicular space) to another (the extravesicular medium).

These findings contrast to some experiments recently reported by Hinkle [22], who also found H^+ release from cytochrome oxidase vesicles on addition of reduced cytochrome *c*, but under conditions of low ionic strength. Under such conditions the acidification phase seemed to be a net effect (contrast above), clearly difficult to relate to a transport process. This acidification, furthermore, showed the odd property of increasing in extent with consecutive cytochrome *c* additions. Under our conditions, the proton ejection is observed in full extent on the first addition of cytochrome *c* and is not subsequently increased (or decreased) in size. Moreover, upon reproducing the results presented by Hinkle [22], we found that the acidification phenomenon is completely insensitive to uncoupling agents under the conditions of low ionic strength (unpublished findings). For these reasons it is evident that this phenomenon has little, if anything, to do with proton transport and with the proton ejection described in this paper. It is possible that the ionic interaction of cytochrome *c* with the phospholipid bilayer surface leads to a net production of H^+ ions at low ionic strength, and it must be considered fortunate that such an effect can be excluded under the experimental conditions used in this paper.

If the proton ejection observed by us is indeed due to redox-linked proton translocation, it must be dependent on the cytochrome *c* oxidase activity. Several control experiments including the effect of cytochrome oxidase inhibitors, addition of cytochrome *c* to vesicles without inlaid enzyme and varying the cytochrome aa_3/phospholipid ratio during vesicle preparation, were fully consistent with this requirement. A redox-linked H^+ translocation process would in addition be expected to show a strict stoichiometric relationship to the "driving" redox reaction. As shown both for recutant and oxidant pulse experiment, measuring initial kinetics of the H^+ ejection and electron

transfer reactions (Figs. 3, 6, 7), a stoichiometry very close to 1 ejected proton per transferred electron was found. This stoichiometry is the same as previously found for cytochrome *c* oxidase-linked proton transport in intact mitochondria and sonicated submitochondrial particles [1,3,4] and provides further support for the notion that the cytochrome *c* oxidase reaction is indeed linked to proton pumping, and for the proposed stoichiometric aspects of this process (see Fig. 1B).

The extent of proton transport across the vesicle membrane would be expected to be limited by the build-up of a pH gradient across the membrane under conditions where K^+ counter-transport catalysed by valinomycin collapses the electrical membrane potential that would otherwise result from the H^+ transport (see below). Such a pH gradient would mainly be due to extensive alkalinisation of the (small) intravesicular space, and would result in H^+ leakage back into the vesicles at a rate proportional to the difference in H^+ concentration between the two aqueous spaces. It is obvious, then, that the magnitude of the pH gradient will depend on (i) the buffering capacity of the intravesicular space, and (ii) on the number of H^+ disappearing from that space. Since the latter is strictly coupled to the redox reaction, it follows that the magnitude of the pH gradient will depend on the number of redox equivalents transferred. The results presented in Fig. 5 are in excellent agreement with these predictions. Thus both the "stability" and the extent (Table I) of the proton ejection were found to show the dependence upon the parameters (i) and (ii) described above and expected from a true transport process. With the above considerations in mind, it is quite obvious that a measurement of the H^+/e^- quotient of proton ejection from the reaction extents (as opposed to the rate determinations discussed above), will be largely underestimating the true H^+/e^- quotient in all cases where the intravesicular buffering capacity is exceeded, resulting in significant backflux of protons into the vesicles. This is quite apparent from the data presented in Table I. However, under optimal conditions with respect to the parameters (i) and (ii), a situation may be achieved where the H^+ disappearing from the intravesicular space are mainly taken from the buffer, without appreciable increase in pH. Such conditions would lead to a minimum of H^+ leakage and may therefore permit an estimation of the true H^+/e^- quotient of proton transport from the reaction extents. Under conditions nearest to this ideal, we observed an H^+/e^- quotient of 0.71 (Table I), which considering the above restrictions must be regarded as corroborating the quotients close to 1.0 observed by the kinetic approach. Due to the problems of using this method to determine the true H^+/e^- quotient, we would tend to dismiss the $H^+/0$ quotients of 2.0 reported by Hinkle [8] for cytochrome oxidase vesicles, pulsed with oxygen in the presence of a hydrogen donor to cytochrome *c*, as underestimates, particularly since no precautions were taken to avoid these difficulties.

An electrogenic proton transport reaction such as that depicted in Fig. 1B would be expected to be associated with generation of an electrical potential difference across the vesicle membrane. Due to the low electrical capacity of the membrane, a very high membrane potential is expected to develop after transport of only very few equivalents of H^+ (cf. refs. 6, 7, 23), much too few to be detected extravesicularly with the presently used techniques. The

generated membrane potential is then expected to "drive" effective proton leakage back into the vesicles, a situation analogous to the one described above for the pH-driven proton leakage. Our finding (see Fig. 4B) of no H^+ ejection in the absence of valinomycin therefore provides strong support for the idea that the cytochrome *c* oxidase reaction is linked to electrogenic proton transport. In the presence of valinomycin, which renders the membranes specifically permeable to K^+, the inward flux of K^+ collapses the membrane potential, whereby proton translocation becomes measurable in the extravesicular space as an ejection of H^+.

We conclude from the several observations reported in this paper and discussed above, that the cytochrome *c* oxidase reaction is most likely linked to true electrogenic proton translocation in a fashion depicted in Fig. 1B. It is significant that we have now provided completely consistent experimental evidence for such a function in intact mitochondria, submitochondrial particles and artificial phospholipid vesicles inlaid with the enzyme. The cytochrome *c* oxidase complex is thus equipped with a proton-pumping device (see refs. 2, 4), which is linked to the redox reaction, and is not merely catalysing electron translocation across the membrane as postulated by Mitchell (see Fig. 1A). Our findings shed serious doubts on the validity of the $H^+/0$ quotients of mitochondrial proton translocation as reported previously using oxidant and reductant pulse techniques [6,7,23—26], but agree with the more recent re-evaluation of these quotients as reported by Brand et al. [27]. However, further careful evaluation of the stoichiometric aspects of redox-linked proton translocation associated with different regions of the respiratory chain, preferably using different independent techniques, seems necessary to definitely resolve the present controversies.

Note added in proof (Received May 29th, 1978)

In a recent communication [28] Moyle and Mitchell have criticised our proposal of a proton-pumping function of cytochrome *c* oxidase. This criticism will be shown to be unfounded [29]. The suggestion by Moyle and Mitchell [28] that extravesicular acidification linked to cytochrome *c* oxidation by cytochrome oxidase vesicles would be an artifactual overall production of H^+ due to protonic dissociation of membraneous acid/base groups interacting with cytochrome *c*, can be excluded by the present data. This suggestion may, however, apply on the net uncoupler-insensitive acidification observed at very low ionic strength (ref. 22 and Discussion).

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